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(54) Title: GENE SILENCING VECTOR

(57) Abstract: The present invention relates to gene silencing methods, more particularly to homology-dependent trans silencing methods. The invention relates to methods for efficiently downregulating any gene in a cell or organism. The invention provides for nucleic acid transgene constructs and vectors for use in said methods. The invention further relates to the use of said constructs or vectors in the production of transgenic cells and transgenic organisms.

GENE SILENCING VECTOR

FIELD OF THE INVENTION

The present invention relates to the field of gene silencing methods. More particularly the present invention relates to the field of homology-dependent trans silencing methods.

BACKGROUND OF THE INVENTION

The phenomenon of "transgene silencing" refers to epigenetic downregulation of an endogenous gene by a transgene inserted in a particular configuration in a particular locus of the genome of an organism. Since transgene silencing requires homology at the nucleic acid sequence level between transgene and silenced endogenous gene, but not a genetic linkage between both loci, the phenomenon is also called "homology-dependent trans silencing" and abbreviated HDTS (Fire A., 1999). Furthermore, as expression of both transgene and homologous endogenous gene is often downregulated in concert, transgene silencing is also referred to as "cosuppression" (Fire A., 1999)

Two types of HDTS can be discriminated, depending on whether the silencing mechanism occurs at the transcriptional level (transcriptional gene silencing, TGS) or post-transcriptional level (post-transcriptional gene silencing, PTGS). TGS generally involves homology between promoter regions of transgene and endogenous gene and is associated with enhanced promoter methylation (Park, Y-D et al, 1996). PTGS, on the other hand, requires homology at the level of the transcripts and involves sequence-specific RNA degradation in the cytoplasm (Depicker A. and Van Montagu M, 1997)

Despite intensive research for about a decade the mechanism underlying PTGS is still not fully understood. One recurrent theme in PTGS appears to be the requirement for double-stranded RNA (dsRNA) as a trigger for sequence-specific RNA degradation. Injection of the nematode *Caenorhabditis elegans* with dsRNA has been shown to result in downregulation of genes homologous to the injected dsRNA (Fire A. *et al* .1998). RNA molecules with dsRNA structures may be recognised by an RNA-dependent RNA polymerase (RdRP) and used as a template to produce copious amounts of antisense RNA. The pivotal role of RdRPs in PTGS is suggested by the observation that a *Neurospora crassa* mutant unable to mount a PTGS response is affected in a RdRP gene (Cogoni C and Macino G, 1999). RdRPs have been shown to be present in plants as well (Schiebel W *et al.* 1998). Antisense RNAs of an average

length of about 25 nucleotides have been detected in plants undergoing PTGS (Hamilton AJ and Baulcombe DC, 1999). Such RNA fragments may hybridize to target RNA and promote sequence specific cleavage of the target RNA, e.g. via a dsRNA-specific nuclease. Direct evidence for the involvement of such nucleases in PTGS has not yet been presented, but it has been observed that PTGS in plants correlates with the appearance of RNA degradation intermediates (van Eldik G. *et al.* 1998)

There is a very high practical potential for HDTS in the area of crop improvement. Genes conferring unwanted traits can be downregulated by transforming the plant with a transgene designed to trigger HDTS. Examples of these types of application are the modification of the content in desaturated lipids by downregulating a fatty acid desaturase ; downregulating fruit ripening genes to improve fruit handling characteristics and extend shelf life ; elimination of toxic secondary metabolites by downregulating a specific biosynthetic gene.

Several research groups have devised strategies for designing transgene constructs such that they efficiently trigger HDTS.

WO98/53083 discloses a method for HDTS based on expression of a transgene which comprises within the transcribed region a subregion homologous to part of the gene targeted for silencing and an inverted repeat of that subregion. The transcripts produced from such transgene are supposed to be capable of forming a structure that is partially double-stranded through base-pairing between the inverted repeat regions. The drawback of this method is the relatively time-consuming and complex construction of the transgene vector with the inverted repeat based on the targeted gene sequence. This technical complexity precludes use of this system for high throughput construction of HDTS vectors.

WO 99/61632 discloses a method that consists in positioning two transgenes (including promoter, transcribed region showing at least partial homology to the targeted gene and a terminator) in an inverted orientation towards each other, yet separated by a spacer sequence. Again, construction of such a vector is cumbersome and time-consuming and not well suited for high throughput analysis of gene downregulation.

WO98/36083 discloses a method for HDTS based on insertion of a region homologous to the transcript of the targeted gene into an "amplicon vector". An amplicon vector comprises a cDNA of a plant virus (or a part thereof) operably flanked at either side by a promoter and a terminator. When such an amplicon vector containing a region homologous to the targeted gene is introduced as a transgene in a plant, viral RNA linked to RNA homologous to the targeted gene will be produced. The viral replication machinery will replicate the transcript, thereby forming positive and negative strands

capable of forming double-stranded RNA. Double stranded RNA homologous to the targeted gene is believed to cause post transcriptional downregulation of the targeted gene. This method requires only relatively simple cloning steps for construction of a HDTS vector and therefore is suitable for high throughput analysis of downregulated genes. A drawback of the method, however, is that it involves viral sequences which may possibly recombine with natural viral sequences, thereby generating new types of plant pathogenic viruses. The method therefore is not to be recommended for applications in which transgenic plants are to be released on a large scale in the environment.

SUMMARY OF THE INVENTION

The present invention aims to overcome the current limitations in the art. The present invention describes a method that allows to efficiently downregulate any gene (whether endogenous gene from the organism or gene from a pathogen of that organism) without making use of viral sequences and without making use of repeat structures in the vector. The absence of repeat structures in the vector facilitates the cloning procedure such that it is compatible with high throughput analysis of gene downregulation via HDTS. Furthermore, the absence of repeat structures in the transgene avoids potential problems of genetic instability of the transgene locus.

Briefly, the present invention consists of incorporating in the genome of an organism (plant, insect, nematode, fungus, vertebrate, etc.) a transgene construct containing the following elements: 1) a promoter capable of driving expression in the organism or tissue of interest and in particular capable of driving expression of the transcription unit to which it is operably linked, 2) a transcribed region of which at least part of the generated transcript is homologous to either the sense or antisense transcript of the targeted gene, 3) a DNA sequence within the transcribed region which consists of a stretch of T bases in the transcribed strand, 4) a DNA sequence immediately following the poly-T stretch which causes transcript termination.

When introduced in the genome of the organism, the transgene construct causes HDTS of the targeted gene. Although the mechanism behind HDTS observed in this case is not known, we believe it may be triggered by double-stranded RNA formation by base-pairing between the internal poly-U stretch of the transcript and the post-transcriptionally added poly-A stretch at the 3' end of the transcript.

A vector which contains the construct may be used in transformation of one or more cells or cells of an organism to introduce the construct stably into the genome, so that it is stably inherited from one generation to the next.

Thus, in further aspects, the present invention also provides the use of the construct or vector in the production of transgenic cells and transgenic organisms.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a nucleic acid construct (transgene) for downregulating the expression of a target nucleic acid in a cell, organism or tissue, comprising the following operably linked elements:

a) a promoter capable of driving expression of said nucleic acid in said cell, organism or tissue,

b) a transcribed region of which at least part of the generated transcript is homologous to either the sense or antisense transcript of the targeted nucleic acid,

c) a DNA sequence within the transcribed region of said nucleic acid which consists of a stretch of T bases in the transcribed strand, and,

d) a DNA sequence immediately following the poly-T stretch which causes transcript termination.

The function of the promoter in the construct is to ensure that the DNA is transcribed into RNA that is in part homologous to the sense or antisense transcript of the targeted gene. By "promoter" is meant a sequence of nucleotides from which transcription may be initiated of DNA operably linked downstream (i.e. in the 3' direction on the sense strand of doublestranded DNA). A promoter "drives" transcription of an operably linked sequence. "Operably linked" means joined as part of the same nucleic acid molecule, suitably positioned and oriented for transcription to be initiated from the promoter. DNA operably linked to a promoter is "under transcriptional initiation regulation" of the promoter, or "in functional combination" therewith.

Preferred promoters for plants may include the 35S promoter of cauliflower mosaic virus or the nopaline synthase promoter of *Agrobacterium tumefaciens* (Sanders, P.R., *et al.*, 1987).

These promoters are expressed in many, if not all cell types of many plants. Other constitutively expressed promoters known in the art may be used effectively as components of the construct for any type of organism or cell. Depending on the target gene, other promoters including those that are developmentally regulated or inducible may be used. For example, if it is necessary to silence the target gene specifically in a particular cell type the construct may be assembled with a promoter that drives transcription only in that cell type. Similarly, if the target gene is to be silenced following a defined external stimulus the construct may incorporate a promoter that is activated

specifically by that stimulus. Promoters that are both tissue specific and inducible by specific stimuli may be used.

A sequence homologous to the targeted gene or nucleic acid may be 500 nucleotides or less, possibly about 400 nucleotides, about 300 nucleotides, about 200 nucleotides or about 100 nucleotides. It may be possible to use oligonucleotides of much shorter lengths, 14-23 nucleotides, although longer fragments, and generally even longer than 500 nucleotides are preferable where possible.

It may be preferably that there is complete sequence identity in the targeted sequence in the construct and the target sequence in the plant or other organism, though total complementarity or similarity of sequence is not essential. Nucleotides in the targeting sequence and the target gene or nucleic acid may differ between 0 and 30 % of the nucleotide positions.

Thus, a targeting sequence employed in a construct in accordance with the present invention may be wild-type sequence (e.g. gene) selected from those available, or a mutant, derivative, variant or allele, by way of insertion, addition, deletion or substitution of one or more nucleotides, of such a sequence. The targeted sequence need not include an open reading frame or specify an RNA that would be translatable. As noted, a foreign sequence may be inserted into the construct in either orientation, for sense or anti-sense regulation. It may be preferred for there to be sufficient homology for the respective anti-sense RNA molecules to hybridise. There may be HDTS even where there is about 5 %, 10 %, 15 %, 20 % or 30 % mismatch between the targeting sequence in the construct and the target gene or nucleic acid.

Advantages of the present invention are the simplicity of the HDTS constructs according to the present invention, the absence of direct or indirect repeats in the gene structure, absence of viral sequences, the reliability and efficiency by which downregulation of gene expression is obtained in transgenic organims and the compatibility of the system proposed according to the present invention with high-throughput cloning and gene function screening methods.

In embodiments of the present invention which have been experimentally exemplified as described below for illustrative and non-limiting purposes only, the foreign sequence in the construct that determined the target of gene silencing was the uidA reporter gene (Jefferson, R. A., et al, 1986). However any other gene of plant, animal, human, nematode, fungal, bacterial or viral origin may be a target of HDTS provided that the corresponding foreign sequence is incorporated into the contruct.

The poly-T stretch in the construct of the invention as described above is positioned such that the poly-T stretch is in the transcribed strand and gives rise to a poly-U

stretch in the transcript. The poly-T stretch may be 500 nucleotides or less, preferably between 10 to 400 nucleotides, more preferably between 20 to 300 nucleotides, even more preferably between 30 and 200 nucleotides, and most preferably between 50 and 100 nucleotides (such as 50, 60, 70, 80, 90 or 100 nucleotides). Said poly-T stretch is preferably located immediately downstream (to the 3'end) of the sequence homologous to the targeted gene. Although less preferred, both regions can be separated by random sequence of undefined length as long as the random sequence does not target an endogenous gene and does not cause transcript termination.

The transcript termination region is positioned immediately downstream of the poly-T stretch region. The transcript termination region can for instance be the transcriptional terminator from the nopaline synthase gene of *Agrobacterium tumefaciens* (Depicker A. *et al*, 1982). Other transcriptional termination regions may be used but it is preferred that the transcribed part of it is not homologous to any of the transcribed sequences of the plant of interest.

The invention further relates to a vector comprising a nucleic acid construct (transgene) of the invention as defined above.

Particularly preferred is a vector which is suitable for stable transformation of a plant cell, such as an Agrobacterium binary vector.

The present invention further provides a vector of the invention as defined above suitable for high-throughput cloning of target nucleic acid sequences, comprising a cloning site for inserting any desired target nucleic acid or a part thereof.

The present invention also relates to a host cell comprising a vector as defined above. Said host cell is for instance a plant, insect, nematode, fungal, plant, human or animal cell.

The present invention also provides a method for downregulating the expression of a target nucleic acid in an organism, tissue or cell comprising the introduction of a vector as defined above into said organism, tissue or cell, resulting in the expression of said nucleic acid construct (transgene) of the invention as described above.

The present method can be used to downregulate or inhibit the expression of genes in the genome of any organism from any taxonomical origin that could receive a transgene according to the present invention. The present invention therefore includes animals (e.g. rats, mice) or animal cells, including human cells (e.g. COS or CHO cells), plants, nematodes, micro-organisms, fungi (e.g. *Saccharomyces cerevisiae, S. pombe, Pichia postoris*) and other, or any part thereof.

Although the examples illustrating the invention relate to the inhibition of gene expression in plants, it is contemplated that the invention can be practised in other organisms too.

When the transgene of the invention has been introduced into an organism, a transgenic organism has been made. Preferably the nucleic acid construct (transgene) is stably added to the genome of the transgenic organism.

The basic principle in the construction of a genetically modified or transgenic organism is to insert genetic information in or to add to the genome so as to obtain stable maintenance of the introduced genetic material. The skilled person will appreciate that transformation of a particular organism can be carried out using techniques well known in the art. The techniques for inserting genetic material depend on the particular organism and are known to experts in the field.

Human germline gene therapy is nevertheless excluded from the scope of the present invention.

The present invention thus also relates to a method of the invention as defined above wherein said nucleotide sequence is stably introduced into the genome of said organism or cell. According to one aspect of the invention said cell is a plant cell.

The present invention also relates to a method of the invention as defined above further comprising regenerating a plant from said plant cell.

The present invention also relates to a method for the production of transgenic plants, plant cells or plant tissues comprising the introduction of a vector of the invention as defined above in an expressible format in said plant, plant cell or plant tissue.

The present invention is applicable to any plant, in particular a monocotyledonous plants and dicotyledonous plants including a fodder or forage legume, ornamental plant, food crop, tree, or shrub selected from the list comprising *Acacia spp., Acer spp., Actinidia spp.,Aesculus spp., Agathis australis, Albizia amara, Alsophila tricolor, Andropogon spp., Arachis spp, Areca catechu, Astelia fragrans, Astragalus cicer, Baikiaea plurijuga, Betula spp., Brassica spp., Bruguiera gymnorrhiza, Burkea africana, Butea frondosa, Cadaba farinosa, Calliandra spp, Camellia sinensis, Canna indica, Capsicum spp., Cassia spp., Centroema pubescens, Chaenomeles spp.,Cinnamomum cassia, Coffea arabica, Colophospermum mopane, Coronillia varia, Cotoneaster serotina, Crataegus spp., Cucumis spp., Cupressus spp., Cyathea dealbata, Cydonia oblonga, Cryptomeria japonica, Cymbopogon spp., Cynthea dealbata, Cydonia oblonga, Dalbergia monetaria, Davallia divaricata, Desmodium spp., Dicksonia squarosa, Diheteropogon amplectens, Dioclea spp, Dolichos spp., Dorycnium rectum, Echinochloa pyramidalis, Ehrartia spp., Eleusine coracana, Eragrestis spp., Erythrina*

spp., Eucalyptus spp., Euclea schimperi, Eulalia villosa, Fagopyrum spp., Feijoa sellowiana, Fragaria spp., Flemingia spp, Freycinetia banksii, Geranium thunbergii, Ginkgo biloba, Glycine javanica, Gliricidia spp, Gossypium hirsutum, Grevillea spp., Guibourtia coleosperma, Hedysarum spp., Hemarthia altissima, Heteropogon contortus, Hordeum vulgare, Hyparrhenia rufa, Hypericum erectum, Hyperthelia dissoluta, Indigo incarnata, Iris spp., Leptarrhena pyrolifolia, Lespediza spp., Lettuca spp., Leucaena leucocephala, Loudetia simplex, Lotonus bainesii, Lotus spp., Macrotyloma axillare, Malus spp., Manihot esculenta, Medicago sativa, Metasequoia glyptostroboides, Musa sapientum, Nicotianum spp., Onobrychis spp., Ornithopus spp., Oryza spp., Peltophorum africanum, Pennisetum spp., Persea gratissima, Petunia spp., Phaseolus spp., Phoenix canariensis, Phormium cookianum, Photinia spp., Picea glauca, Pinus spp., Pisum sativum, Podocarpus totara, Pogonarthria fleckii, Pogonarthria squarrosa, Populus spp., Prosopis cineraria, Pseudotsuga menziesii, Pterolobium stellatum, Pyrus communis, Quercus spp., Rhaphiolepsis umbellata, Rhopalostylis sapida, Rhus natalensis, Ribes grossularia, Ribes spp., Robinia pseudoacacia, Rosa spp., Rubus spp., Salix spp., Schyzachyrium sanguineum, Sciadopitys verticillata, Sequoia sempervirens, Sequoiadendron giganteum, Sorghum bicolor, Spinacia spp., Sporobolus fimbriatus, Stiburus alopecuroides, Stylosanthos humilis, Tadehagi spp, Taxodium distichum, Themeda triandra, Trifolium spp., Triticum spp., Tsuga heterophylla, Vaccinium spp., Vicia spp.Vitis vinifera, Watsonia pyramidata, Zantedeschia aethiopica, Zea mays, amaranth, artichoke, asparagus, broccoli, brussel sprout, cabbage, canola, carrot, cauliflower, celery, collard greens, flax, kale, lentil, oilseed rape, okra, onion, potato, rice, soybean, straw, sugarbeet, sugar cane, sunflower, tomato, squash, and tea, amongst others, or the seeds of any plant specifically named above or a tissue, cell or organ culture of any of the above species.

The present invention further relates to a transgenic plant cell comprising a nucleic acid construct of the invention as defined above or obtainable by a method of the invention as defined above.

The present invention also relates to a transgenic plant cell of the invention as defined above wherein said nucleic acid construct of the invention as defined above is stably integrated into the genome of said plant cell.

The present invention also relates to a transgenic plant or plant tissue comprising plant cells of the invention as defined above or obtainable by a method of the invention as defined above. The present invention also provides a harvestable part or extract or

derivative of said plant. Said harvestable part is for instance selected from the group consisting of seeds, fruits, leaves, stem cultures, rhizomes and bulbs.

The present invention also relates to the progeny derived from any of the plants or plant parts of the invention as defined above.

The present invention also relates to a method for modifying cell fate comprising the use of a method of the invention as defined above.

The present invention also relates to a method for modifying the development of a whole organism, tissue or organ comprising the use of a method of the invention as defined above.

The present invention also relates to a method for modifying the morphology of a whole organism, tissue or organ comprising the use of a method of the invention as defined above.

The present invention also relates to a method for modifying the biochemistry of a whole organism, tissue or organ comprising the use of a method of the invention as defined above.

The present invention also relates to a method for modifying the physiology of a whole organism, tissue or organ comprising the use of a method of the invention as defined above.

The present invention also relates to a method for modifying the cell cycle progression rate of a whole organism, tissue or organ comprising the use of a method of the invention as defined above.

The present invention also relates to a method for obtaining enhanced growth of a whole organism, tissue or organ comprising the use of a method of the invention as defined above.

The present invention also relates to a method for obtaining increased yield of a plant or harvestable part thereof comprising the use of a method of the invention as defined above.

The present invention also relates to a method for obtaining delayed senescence of a whole plant, a plant tissue or organ comprising the use of a method of the invention as defined above.

The present invention also relates to a method for modifying the content of desaturated lipids of a plant by downregulating a fatty acid desaturase comprising the use of a method of the invention as defined above.

The present invention also relates to a method for improving fruit handling characteristics by downregulating fruit ripening genes comprising the use of a method of the invention as defined above.

The present invention also relates to a method for elimination of toxic secondary metabolites in a plant by downregulating a biosynthetic gene comprising the use of a method of the invention as defined above.

The present invention also relates to a method for promoting and extending cell division activity in cells or organisms in adverse conditions and/or consequences of stress, comprising the use of a method of the invention as defined above.

The present invention also relates to a method for conferring enhanced resistance to pathogens comprising the use of a method of the invention as defined above. In the case of plants such pathogens may be plant pathogenic bacteria including *Agrobacterium tumefaciens*, plant pathogenic fungi including *Plasmodiophora brassicae, Crinipellis perniciosa, Pucciniastrum geoppertianum, Taphrina wiesneri, Ustilaga maydis, Exobasidium vaccinii, E. camelliae, Entorrhiza casparyana* and *Apiosporina morbosum.*

The present invention also relates to a method for conferring enhanced resistance to pathogens causing neoplastic growth comprising the use of a method of the invention as defined above.

The present invention also relates to a method for increasing seed yield and/or seed size comprising the use of a method of the invention as defined above.

The present invention relates to a method for identifying the function of a target nucleic acid in an organism, cell, tissue or organ, comprising elucidating the phenotypes of the organism, cell, organ or tissue which transcribe a nucleic acid construct of the invention as defined above.

According to this aspect, the present invention relates to elucidating the function of genes with hitherto unknown roles as well as to elucidating new functions of genes with an already known or presumed function. The present invention also allows the function of a group of homologous or partially homologous genes which may be redundant or overlapping in biological function, to be evaluated.

Said target nucleic acid in said method may be a gene, a cDNA or a part of said gene or cDNA. Said part of said cDNA may be an expressed sequence tag (EST).

The present invention particularly relates to a method of the invention as defined here above further characterized as being a high-throughput cloning method for identifying the function of target nucleic acids, comprising the use of a vector of the invention as defined above. Said organism of said method may be a plant, insect, nematode, fungus, or a non-human animal.

The present invention also relates to the use of a nucleic construct of claim of the invention as defined above or a vector of claim of the invention as defined above for modifying cell fate.

The present invention also relates to the use of a nucleic construct of claim of the invention as defined above or a vector of the invention as defined above for modifying development of an organism.

The present invention also relates to the use of a nucleic acid construct of the invention as defined above or a vector of the invention as defined above, for modifying morphology of an organism.

The present invention also relates to the use of a nucleic acid construct of the invention as defined above or a vector of the invention as defined above for modifying plant biochemistry.

The present invention also relates to the use of a nucleic acid construct of the invention as defined above or a vector of the invention as defined above for modifying plant physiology.

The present invention also relates to a nucleic acid construct of the invention as defined above or a vector of the invention as defined above for use as a medicament.

The present invention also relates to the use of a nucleic acid construct of the invention as defined above or a vector of the invention as defined above for the preparation of a medicament for treating infectious diseases, tumors, auto-immune diseases, diseases due to organ gland or tissue malfunctioning, diseases due to biochemical malfunctioning, transplant rejection reactions or septic shock in animals or humans.

The present invention also relates to a composition comprising a nucleic acid construct of the invention as defined above or a vector of the invention as defined above a host cell of the invention as defined above.

DEFINITIONS AND ELABORATIONS TO THE EMBODIMENTS

The manipulation of DNA sequences to produce sequences homologous to the targeted gene or nucleic acid which are substitutional, insertional or deletional variants of the targeted gene or nucleic acid are well known in the art. For example, techniques for making substitution mutations at predetermined sites in DNA having known sequence are well known to those skilled in the art, such as by M13 mutagenesis, T7-Gen in vitro mutagenesis kit (USB, Cleveland, OH), QuickChange Site Directed mutagenesis kit (Stratagene, San Diego, CA), PCR-mediated site-directed mutagenesis or other site-directed mutagenesis protocols.

The terms "target gene(s)", "target polynucleotide(s)", "target nucleic acid construct(s)", "target nucleotide sequence(s)", or "target nucleic acid(s)", when used herein refer to nucleotides, either ribonucleotides or deoxyribonucleotides or a combination of both, in a polymeric form of any length. Said terms furthermore include double-stranded and single-stranded DNA and RNA. Said double-stranded DNA molecules include genomic DNA or cDNA. Said cDNA molecules also include expressed sequence tags or ESTs.

A "coding sequence" or "open reading frame" or "ORF" is defined as a nucleotide sequence that can be transcribed into mRNA and/or translated into a polypeptide when placed under the control of appropriate regulatory sequences, i.e. when said coding sequence or ORF is present in an expressible format. Said coding sequence of ORF is bounded by a 5' translation start codon and a 3' translation stop codon. A coding sequence or ORF can include, but is not limited to RNA, mRNA, cDNA, recombinant nucleotide sequences, synthetically manufactured nucleotide sequences or genomic DNA. Said coding sequence or ORF can be interrupted by intervening nucleic acid sequences.

With "vector" or "vector sequence" is meant a DNA sequence which can be introduced in an organism by transformation and can be stably maintained in said organism. Vector maintenance is possible in e.g. cultures of *Escherichia coli*, *A. tumefaciens*, *Saccharomyces cerevisiae* or *Schizosaccharomyces pombe*. Other vectors such as phagemids and cosmid vectors can be maintained and multiplied in bacteria and/or viruses. Vector sequences generally comprise a set of unique sites recognized by restriction enzymes, the multiple cloning site (MCS); wherein one or more non-vector sequence(s) can be inserted.

A vector may advantageously be a plasmid, cosmid, virus or other suitable vector which would be known to those skilled in the art.

In the context of the present invention, preferred host cells are eukaryotic cells, yeast cells or bacterial cells. Preferred eukaryotic cells are plant cells mammalian cell or insect cells. Mammalian host cells are particularly advantageous because they provide the necessary post-translational modifications to the expressed proteins according to the invention, such as glycosylation or acetylation or the like, which modifications confer optimal biological activity on said proteins, which when isolated may advantageously be used in diagnostic kits or the like. Insect host cells are particularly advantageous because they allow the use of a recombinant baculovirus expression system, which would provide high yields of recombinant huHDAC4 protein.

With "non-vector sequence" is accordingly meant a DNA sequence which is integrated in one or more of the sites of the MCS comprised within a vector.

By "expressible format" is meant that the isolated nucleic acid molecule is in a form suitable for being transcribed into mRNA, either constitutively or following induction by an intracellular or extracellular signal, such as in the case of a plant or plant cell an environmental stimulus or stress (mitogens, anoxia, hypoxia, temperature, salt, light, dehydration, etc) or a chemical compound such as IPTG (isopropyl-β-D-thiogalactopyranoside) or such as an antibiotic (tetracycline, ampicillin, rifampicin, kanamycin), hormone (e.g. gibberellin, auxin, cytokinin, glucocorticoid, brassinosteroid, ethylene, abscisic acid etc), hormone analogue (iodoacetic acid (IAA), 2,4-D, etc) , metal (zinc, copper, iron, etc), or dexamethasone, amongst others.

In the context of the present invention, the promoter may be a promoter sequence that functions or solely functions in plant cells, bacteria, yeast cells, insect cells, nematodes, animal cells or human cells. All these promoters are well known in the art.

Reference herein to a "promoter" according to the present invention is to be taken in its broadest context and includes the transcriptional regulatory sequences derived from a classical eukaryotic genomic gene, including the TATA box which is required for accurate transcription initiation, with or without a CCAAT box sequence and additional regulatory elements (i.e. upstream activating sequences, enhancers and silencers) which alter gene expression in response to developmental and/or external stimuli, or in a tissue-specific manner.

The term "promoter" also includes the transcriptional regulatory sequences of a classical prokaryotic gene, in which case it may include a -35 box sequence and/or a -10 box transcriptional regulatory sequences.

The term "promoter" is also used to describe a synthetic or fusion molecule, or derivative which confers, activates or enhances expression of a nucleic acid molecule in a cell, tissue or organ.

Promoters may contain additional copies of one or more specific regulatory elements, to further enhance expression and/or to alter the spatial expression and/or temporal expression of a nucleic acid molecule to which it is operably connected. Such regulatory elements may be placed adjacent to a heterologous promoter sequence to drive expression of a nucleic acid molecule in response to e.g. in the case of plant promoters: copper, glucocorticoids, dexamethasone, tetracycline, gibberellin, cAMP, abscisic acid, auxin, wounding, ethylene, jasmonate or salicylic acid; or to confer expression of a nucleic acid molecule to specific cells, tissues or organs such as meristems, leaves, roots, embryo, flowers, seeds or fruits.

By "plant-expressible" is meant that the promoter sequence, including any additional regulatory elements added thereto or contained therein, is at least capable of inducing, conferring, activating or enhancing expression in a plant cell, tissue or organ, preferably a monocotyledonous or dicotyledonous plant cell, tissue, or organ.

Regulatable promoters as part of a binary viral plant expression system are also known to the skilled artisan (Yadav 1999 – WO 9922003; Yadav 2000 – WO 0017365).

The terms "plant-operable" and "operable in a plant" when used herein, in respect of a promoter sequence, shall be taken to be equivalent to a plant-expressible promoter sequence.

In the present context, a "regulatable promoter sequence" is a promoter that is capable of conferring expression on a structural gene in a particular cell, tissue, or organ or group of cells, tissues or organs of an organism, optionally under specific conditions, however does generally not confer expression throughout the organism under all conditions. Accordingly, a regulatable promoter sequence may be a promoter sequence that confers expression on a gene to which it is operably connected in a particular location within the organism or alternatively, throughout the organism under a specific set of conditions, such as following induction of gene expression by a chemical compound or other elicitor.

According to one aspect, the regulatable plant promoter used in the performance of the present invention confers expression in a specific location within the plant, either constitutively or following induction, however not in the whole plant under any circumstances. Included within the scope of such promoters are cell-specific promoter sequences, tissue-specific promoter sequences, organ-specific promoter sequences, cell cycle specific gene promoter sequences, inducible promoter sequences and constitutive promoter sequences that have been modified to confer expression in a particular part of the plant at any one time, such as by integration of said constitutive promoter within a transposable genetic element (*Ac, Ds, Spm, En*, or other transposon).

The term "cell-specific" shall be taken to indicate that expression is predominantly in a particular cell or cell-type, possibly of plant origin, albeit not necessarily exclusively in said cell or cell-type.

Similarly, the term "tissue-specific" shall be taken to indicate that expression is predominantly in a particular tissue or tissue-type, possibly of plant origin, albeit not necessarily exclusively in said tissue or tissue-type.

Similarly, the term "organ-specific" shall be taken to indicate that expression is predominantly in a particular organ, possibly of plant origin, albeit not necessarily exclusively in said organ.

Similarly, the term "cell cycle specific" shall be taken to indicate that expression is predominantly cyclic and occurring in one or more, not necessarily consecutive phases of the cell cycle albeit not necessarily exclusively in cycling cells, possibly of plant origin.

Those skilled in the art will be aware that an "inducible promoter" is a promoter the transcriptional activity of which is increased or induced in response to a developmental, chemical, environmental, or physical stimulus. Similarly, the skilled craftsman will understand that a "constitutive promoter" is a promoter that is transcriptionally active throughout most, but not necessarily all parts of an organism, during most, but not necessarily all phases of its growth and development.

Those skilled in the art will readily be capable of selecting appropriate promoter sequences for use in regulating appropriate expression from publicly-available or readily-available sources, without undue experimentation.

Placing a nucleic acid molecule under the regulatory control of a promoter sequence, or in operable connection with a promoter sequence, means positioning said nucleic acid molecule such that expression is controlled by the promoter sequence. A promoter is usually, but not necessarily, positioned upstream, or at the 5'-end, and within 2 kb of the start site of transcription, of the nucleic acid molecule which it regulates. In the construction of heterologous promoter/structural gene combinations it is generally preferred to position the promoter at a distance from the gene transcription start site that is approximately the same as the distance between that promoter and the gene it controls in its natural setting (i.e., the gene from which the promoter is derived). As is known in the art, some variation in this distance can be accommodated without loss of promoter function. Similarly, the preferred positioning of a regulatory sequence element with respect to a heterologous gene to be placed under its control is defined by the positioning of the element in its natural setting (i.e., the gene from which it is derived). Again, as is known in the art, some variation in this distance can also occur.

Examples of plant promoters suitable for use in gene constructs of the present invention include those listed in Table 1, amongst others. The promoters listed in Table 1 are provided for the purposes of exemplification only and the present invention is not to be limited by the list provided therein. Those skilled in the art will readily be in a position to provide additional promoters that are useful in performing the present invention.

In the case of constitutive promoters or promoters that induce expression throughout the entire plant, it is preferred that such sequences are modified by the addition of nucleotide sequences derived from one or more of the tissue-specific promoters listed in Table 1, or alternatively, nucleotide sequences derived from one or more of the above-mentioned tissue-specific inducible promoters, to confer tissue-specificity thereon. For example, the CaMV 35S promoter may be modified by the addition of maize *Adh1* promoter sequence, to confer anaerobically-regulated root-specific expression thereon, as described previously (Ellis *et al.*, 1987). Another example describes conferring root specific or root abundant gene expression by fusing the CaMV35S promoter to elements of the maize glycin-rich protein GRP3 gene (Feix and Wulff 2000 - WO0015662). Such modifications can be achieved by routine experimentation by those skilled in the art.

The term "terminator" refers to a DNA sequence at the end of a transcriptional unit which signals termination of transcription. Terminators are 3'-non-translated DNA sequences containing a polyadenylation signal, which facilitates the addition of polyadenylate sequences to the 3'-end of a primary transcript. Terminators active in cells derived from viruses, yeasts, moulds, bacteria, insects, birds, nematodes, mammals and plants are known and described in the literature. They may be isolated from these organisms.

Examples of terminators particularly suitable for use in the plant gene constructs of the present invention include the *Agrobacterium tumefaciens* nopaline synthase (NOS) gene terminator, the *Agrobacterium tumefaciens* octopine synthase (OCS) gene terminator sequence, the Cauliflower mosaic virus (CaMV) 35S gene terminator sequence, the *Oryza sativa* ADP-glucose pyrophosphorylase terminator sequence (t3'Bt2), the *Zea mays* zein gene terminator sequence, the *rbcs-1A* gene terminator, and the *rbcs-3A* gene terminator sequences, amongst others.

Table 1. Exemplary plant-expressible promoters for use in the performance of the present invention

I: CELL-SPECIFIC,TISSUE-SPECIFIC, AND ORGAN-SPECIFIC PROMOTERS		
GENE SOURCE	**EXPRESSION PATTERN**	**REFERENCE**
α-amylase (*Amy32b*)	aleurone	Lanahan, M.B., *e t al., Plant Cell 4:*203-211, 1992; Skriver, K., *et al. Proc. Natl. Acad. Sci. (USA) 88:* 7266-7270, 1991
cathepsin β-like gene	aleurone	Cejudo, F.J., *et al. Plant Molecular Biology 20:*849-856, 1992.
Agrobacterium rhizogenes rolB	cambium	Nilsson *et al., Physiol. Plant. 100:*456-462, 1997
AtPRP4	flowers	http://salus.medium.edu/mmg/tierney/html
chalcone synthase (chsA)	flowers	Van der Meer, *et al., Plant Mol. Biol. 15*, 95-109, 1990.
LAT52	anther	Twell et al Mol. Gen Genet. 217:240-245 (1989)
apetala-3	flowers	
chitinase	fruit (berries, grapes, etc)	Thomas *et al.* CSIRO Plant Industry, Urrbrae, South Australia, Australia; http://winetitles.com.au/gwrdc/csh95-1.html
rbcs-3A	green tissue (eg leaf)	Lam, E. *et al., The Plant Cell 2:* 857-866, 1990.; Tucker *et al., Plant Physiol. 113:* 1303-1308, 1992.
leaf-specific genes	leaf	Baszczynski, *et al., Nucl. Acid Res. 16:* 4732, 1988.
AtPRP4	leaf	http://salus.medium.edu/mmg/tierney/html
chlorella virus adenine methyltransferase gene promoter	leaf	Mitra and Higgins, 1994, Plant Molecular Biology 26: 85-93
aldP gene promoter from rice	leaf	Kagaya et al., 1995, Molecular and General Genetics 248: 668-674
rbcs promoter from rice or tomato	leaf	Kyozuka et al., 1993, Plant Physiology 102: 991-1000
Pinus cab-6	leaf	Yamamoto *et al., Plant Cell Physiol. 35:*773-778, 1994.
rubisco promoter	leaf	
cab (chlorophyll a/b/binding protein	leaf	
SAM22	senescent leaf	Crowell, *et al., Plant Mol. Biol. 18:*

		459-466, 1992.
ltp gene (lipid transfer gene)		*Fleming, et al, Plant J. 2, 855-862.*
R. japonicum nif gene	nodule	United States Patent No. 4, 803, 165
B. japonicum nifH gene	nodule	United States Patent No. 5, 008, 194
GmENOD40	nodule	Yang, *et al., The Plant J. 3:* 573-585.
PEP carboxylase (PEPC)	nodule	Pathirana, *et al., Plant Mol. Biol. 20:* 437-450, 1992.
leghaemoglobin (Lb)	nodule	Gordon, *et al., J. Exp. Bot. 44:* 1453-1465, 1993.
Tungro bacilliform virus gene	phloem	Bhattacharyya-Pakrasi, *et al, The Plant J. 4:* 71-79, 1992.
pollen-specific genes	pollen; microspore	Albani, *et al., Plant Mol. Biol. 15:* 605, 1990; Albani, *et al., Plant Mol. Biol. 16:* 501, 1991)
Zm13	pollen	Guerrero et al Mol. Gen. Genet. 224:161-168 (1993)
apg gene	microspore	Twell et al Sex. Plant Reprod. 6:217-224 (1993)
maize pollen-specific gene	pollen	Hamilton, et al., Plant Mol. Biol. 18: 211-218, 1992.
sunflower pollen-expressed gene	pollen	Baltz, et al., The Plant J. 2: 713-721, 1992.
B. napus pollen-specific gene	pollen;anther; tapetum	Arnoldo, et al., J. Cell. Biochem., Abstract No. Y101, 204, 1992.
root-expressible genes	roots	Tingey, et al., EMBO J. 6: 1, 1987.
tobacco auxin-inducible gene	root tip	Van der Zaal, et al., Plant Mol. Biol. 16, 983, 1991.
β-tubulin	root	Oppenheimer, *et al., Gene 63:* 87, 1988.
tobacco root-specific genes	root	Conkling, et al., Plant Physiol. 93: 1203, 1990.
B. napus G1-3b gene	root	United States Patent No. 5, 401, 836
SbPRP1	roots	Suzuki et al., Plant Mol. Biol. 21: 109-119, 1993.
AtPRP1; AtPRP3	roots; root hairs	http://salus.medium.edu/mmg/tierney/html
RD2 gene	root cortex	http://www2.cnsu.edu/ncsu/research
TobRB7 gene	root vasculature	http://www2.cnsu.edu/ncsu/research
AtPRP4	leaves; flowers; lateral root primordia	http://salus.medium.edu/mmg/tierney/html
seed-specific genes	seed	Simon, *et al., Plant Mol. Biol. 5:* 191, 1985; Scofield, *et al., J. Biol. Chem. 262:* 12202, 1987.; Baszczynski, *et al., Plant Mol. Biol. 14:* 633, 1990.

Brazil Nut albumin	seed	Pearson, et al., Plant Mol. Biol. 18: 235-245, 1992.
legumin	seed	Ellis, et al., Plant Mol. Biol. 10: 203-214, 1988.
glutelin (rice)	seed	Takaiwa, et al., Mol. Gen. Genet. 208: 15-22, 1986; Takaiwa, et al., FEBS Letts. 221: 43-47, 1987.
zein	seed	Matzke et al Plant Mol Biol, 14(3):323-32 1990
napA	seed	Stalberg, *et al, Planta 199:* 515-519, 1996.
wheat LMW and HMW glutenin-1	endosperm	Mol Gen Genet 216:81-90, 1989; NAR 17:461-2, 1989
wheat SPA	seed	Albani *et al,* Plant Cell, 9: 171-184, 1997
wheat α, β, γ-gliadins	endosperm	EMBO 3:1409-15, 1984
barley *ltr1* promoter	endosperm	
barley B1, C, D, hordein	endosperm	Theor Appl Gen 98:1253-62, 1999; Plant J 4:343-55, 1993; Mol Gen Genet 250:750-60, 1996
barley DOF	endosperm	Mena *et al,* The Plant Journal, 116(1): 53-62, 1998
blz2	endosperm	EP99106056.7
synthetic promoter	endosperm	Vicente-Carbajosa *et al., Plant J. 13:* 629-640, 1998.
rice prolamin NRP33	endosperm	Wu *et al,* Plant Cell Physiology 39(8) 885-889, 1998
rice α-globulin Glb-1	Endosperm	Wu *et al,* Plant Cell Physiology 39(8) 885-889, 1998
rice OSH1	embryo	Sato *et al,* Proc. Natl. Acad. Sci. USA, 93: 8117-8122, 1996
rice α-globulin REB/OHP-1	endosperm	Nakase *et al.* Plant Mol. Biol. 33: 513-522, 1997
rice ADP-glucose PP	endosperm	Trans Res 6:157-68, 1997
maize ESR gene family	endosperm	Plant J 12:235-46, 1997
sorgum γ-kafirin	endosperm	PMB 32:1029-35, 1996
KNOX	embryo	Postma-Haarsma *et al,* Plant Mol. Biol. 39:257-71, 1999
rice oleosin	embryo and aleuron	Wu *et at,* J. Biochem., 123:386, 1998
sunflower oleosin	seed (embryo and dry seed)	Cummins, *et al.*, Plant Mol. Biol. 19: 873-876, 1992
LEAFY	shoot meristem	Weigel *et al., Cell 69*:843-859, 1992.
Arabidopsis thaliana knat1	shoot meristem	Accession number AJ131822
Malus domestica kn1	shoot meristem	Accession number Z71981
CLAVATA1	shoot meristem	Accession number AF049870

stigma-specific genes	stigma	Nasrallah, et al., Proc. Natl. Acad. Sci. USA 85: 5551, 1988; Trick, *et al., Plant Mol. Biol. 15:* 203, 1990.
class I patatin gene	tuber	Liu et al., Plant Mol. Biol. 153:386-395, 1991.
PCNA rice	meristem	Kosugi *et al, Nucleic Acids Research 19:*1571-1576, 1991; Kosugi S. and Ohashi Y, *Plant Cell 9:*1607-1619, 1997.
Pea TubA1 tubulin	Dividing cells	Stotz and Long, *Plant Mol.Biol.* 41, 601-614. 1999
Arabidopsis cdc2a	cycling cells	Chung and Parish, FEBS Lett, 3;362(2):215-9, 1995
Arabidopsis Rop1A	Anthers; mature pollen + pollen tubes	Li et al. 1998 *Plant Physiol* 118, 407-417.
Arabidopsis AtDMC1	Meiosis-associated	Klimyuk and Jones 1997 *Plant J.* 11, 1-14.
Pea PS-IAA4/5 and PS-IAA6	Auxin-inducible	Wong et al. 1996 *Plant J.* 9, 587-599.
Pea farnesyltransferase	Meristematic tissues; phloem near growing tissues; light- and sugar-repressed	Zhou et al. 1997 *Plant J.* 12, 921-930
Tobacco (*N. sylvestris*) cyclin B1;1	Dividing cells / meristematic tissue	Trehin et al. 1997 *Plant Mol.Biol.* 35, 667-672.
Catharanthus roseus Mitotic cyclins CYS (A-type) and CYM (B-type)	Dividing cells / meristematic tissue	Ito et al. 1997 *Plant J.* 11, 983-992
Arabidopsis cyc1At (=cyc B1;1) and cyc3aAt (A-type)	Dividing cells / meristematic tissue	Shaul et al. 1996 *Proc.Natl.Acad.Sci.U.S.A* 93, 4868-4872.
Arabidopsis tef1 promoter box	Dividing cells / meristematic tissue	Regad et al. 1995 *Mol.Gen.Genet.* 248, 703-711.
Catharanthus roseus cyc07	Dividing cells / meristematic tissue	Ito et al. 1994 *Plant Mol.Biol.* 24, 863-878.

Table 1 (continued). Exemplary plant-expressible promoters for use in the performance of the present invention

II: EXEMPLARY CONSTITUTIVE PROMOTERS		
GENE SOURCE	**EXPRESSION PATTERN**	**REFERENCE**
Actin	constitutive	McElroy *et al,* Plant Cell, 2: 163-171, 1990
CAMV 35S	constitutive	Odell *et al,* Nature, 313: 810-812, 1985
CaMV 19S	constitutive	Nilsson *et al.,* Physiol. Plant. 100:456-462, 1997
GOS2	constitutive	de Pater *et al,* Plant J. 2:837-844, 1992
ubiquitin	constitutive	Christensen *et al,* Plant Mol. Biol. 18: 675-689, 1992
rice cyclophilin	constitutive	Buchholz *et al,* Plant Mol Biol. 25: 837-843, 1994
maize histone H3	constitutive	Lepetit *et al,* Mol. Gen. Genet. 231:276-285, 1992
alfalfa histone H3	constitutive	Wu *et al.*, Nucleic Acids Res. 17: 3057-3063, 1989; Wu *et al.*, Plant Mol. Biol. 11:641-649, 1988
actin 2	constitutive	An *et al,* Plant J. 10(1); 107-121, 1996
III: EXEMPLARY STRESS-INDUCIBLE PROMOTERS		
NAME	**STRESS**	**REFERENCE**
P5CS (delta(1)-pyrroline-5-carboxylate syntase)	salt, water	Zhang et al. Plant Science. 129: 81-89, 1997
cor15a	cold	Hajela et al., Plant Physiol. 93: 1246-1252, 1990
cor15b	cold	Wlihelm et al., Plant Mol Biol. 23:1073-1077, 1993
cor15a (-305 to +78 nt)	cold, drought	Baker et al., Plant Mol Biol. 24: 701-713, 1994
rd29	salt, drought, cold	Kasuga et al., Nature Biotechnology 18:287-291, 1999
heat shock proteins, including artificial promoters containing the heat shock element (HSE)	heat	Barros et al., Plant Mol Biol 19: 665-75, 1992. Marrs et al., Dev Genet.14: 27-41, 1993. Schoffl et al., Mol Gen Gent, 217: 246-53, 1989.
smHSP (small heat shock proteins)	heat	Waters et al, J Experimental Botany 47:325-338, 1996
wcs120	cold	Ouellet et al., FEBS Lett. 423: 324-328, 1998
ci7	cold	Kirch et al., Plant Mol Biol 33: 897-909, 1997

Adh	cold, drought, hypoxia	Dolferus et al., Plant Physiol 105: 1075-87, 1994
pwsi18	water: salt and drought	Joshee et al., Plant Cell Physiol 39: 64-72, 1998
ci21A	cold	Schneider et al., Plant Physiol 113: 335-45, 1997
Trg-31	drought	Chaudhary et al., Plant Mol Biol 30: 1247-57, 1996
osmotin	osmotic	Raghothama et al., Plant Mol Biol 23: 1117-28, 1993
lapA	wounding, enviromental	WO99/03977 University of California/INRA
IV: EXEMPLARY PATHOGEN-INDUCIBLE PROMOTERS		
NAME	**PATHOGEN**	**REFERENCE**
RB7	Root-knot nematodes (Meloidogyne spp.)	US5760386 - North Carolina State University; Opperman et al (1994) *Science* 263: 221-23.
PR-1, 2, 3, 4, 5, 8, 11	fungal, viral, bacterial	Ward et al (1991) Plant Cell 3: 1085-1094; Reiss et al 1996; Lebel et al (1998), Plant J, 16(2):223-33; Melchers et al (1994), Plant J, 5(4):469-80; Lawton et al (1992), Plant Mol Biol, 19(5):735-43.
HMG2	nematodes	WO9503690 - Virginia Tech Intellectual Properties Inc .
Abi3	Cyst nematodes (Heterodera spp.)	Unpublished
ARM1	nematodes	Barthels et al., (1997) The Plant Cell 9, 2119-2134. WO 98/31822 – Plant Genetic Systems
Att0728	nematodes	Barthels et al., (1997) The Plant Cell 9, 2119-2134. PCT/EP98/07761
Att1712	nematodes	Barthels et al., (1997) The Plant Cell 9, 2119-2134. PCT/EP98/07761
Gst1	Different types of pathogens	Strittmatter et al (1996) Mol. Plant-Microbe Interact. 9, 68-73.
LEMMI	nematodes	WO 92/21757 – Plant Genetic Systems
CLE	geminivirus	PCT/EP99/03445 - CINESTAV
PDF1.2	Fungal including *Alternaria brassicicola* and *Botrytis cinerea*	Manners et al (1998), Plant Mol Biol, 38(6):1071-80.
Thi2.1	Fungal – *Fusarium oxysporum f sp. matthiolae*	Vignutelli et al (1998) Plant J;14(3):285-95
DB#226	nematodes	Bird and Wilson (1994) Mol. Plant-

		Microbe Interact., 7, 419-42 WO 95.322888
DB#280	nematodes	Bird and Wilson (1994) Mol. Plant-Microbe Interact., 7, 419-42 WO 95.322888
Cat2	nematodes	Niebel et al (1995) Mol Plant Microbe Interact 1995 May-Jun;8(3):371-8
□Tub	nematodes	Aristizabal et al (1996), 8th International Congress on Plant-Microbe Interaction, Knoxville US B-29
SHSP	nematodes	Fenoll et al (1997) In: Cellular and molecular aspects of plant-nematode interactions. Kluwer Academic, C. Fenoll, F.M.W. Grundler and S.A. Ohl (Eds.),
Tsw12	nematodes	Fenoll et al (1997) In: Cellular and molecular aspects of plant-nematode interactions. Kluwer Academic, C. Fenoll, F.M.W. Grundler and S.A. Ohl (Eds.)
Hs1(pro1)	nematodes	WO 98/122335 - Jung
NsLTP	viral, fungal, bacterial	Molina & Garc´ia-Olmedo (1993) FEBS Lett, 316(2):119-22
RIP	viral, fungal	Tumer et al (1997) Proc Natl Acad Sci U S A, 94(8):3866-71

Those skilled in the art will be aware of additional promoter sequences and terminator sequences which may be suitable for use in performing the invention. Such sequences may readily be used without any undue experimentation.

By "modifying cell fate and/or development and/or morphology and/or biochemistry and/or physiology" is meant that one or more developmental and/or morphological and/or biochemical and/or physiological characteristics of a plant, fungus, yeast, nematode, animal or insect is altered by the performance of one or more steps pertaining to the invention described herein.

"Cell fate" refers to the cell-type or cellular characteristics of a particular cell that are produced during development of an organism or a cellular process therefore, in particular during the cell cycle or as a consequence of a cell cycle process.

"Development" or the term "developmental characteristic" or a similar term shall, when used herein, be taken to mean any cellular process of an organism that is involved in determining the developmental fate of a cell, in particular the specific tissue or organ type into which a progenitor cell will develop. Cellular processes relevant to development will be known to those skilled in the art. Such processes include, for

example, in plants, morphogenesis, photomorphogenesis, shoot development, root development, vegetative development, reproductive development, stem elongation, flowering, and regulatory mechanisms involved in determining cell fate, in particular a process or regulatory process involving the cell cycle.

"Morphology" or the term "morphological characteristic" or a similar term will, when used herein, be understood by those skilled in the art to refer to the external appearance of an organism such as a plant, animal of nematode, including any one or more structural features or combination of structural features thereof. Such structural features include the shape, size, number, position, colour, texture, arrangement, and patternation of any cell, tissue or organ or groups of cells, tissues or organs, including for plants, the root, stem, leaf, shoot, petiole, trichome, flower, petal, stigma, style, stamen, pollen, ovule, seed, embryo, endosperm, seed coat, aleurone, fibre, fruit, cambium, wood, heartwood, parenchyma, aerenchyma, sieve element, phloem or vascular tissue, amongst others.

"Biochemistry" or the term "biochemical characteristic" or a similar term will, when used herein, be understood by those skilled in the art to refer to the metabolic and catalytic processes of an organism, including in case of a plant primary and secondary metabolism and the products thereof, including any small molecules, macromolecules or chemical compounds, such as but not limited to starches, sugars, proteins, peptides, enzymes, hormones, growth factors, nucleic acid molecules, celluloses, hemicelluloses, calloses, lectins, fibres, pigments such as anthocyanins, vitamins, minerals, micronutrients, or macronutrients, that are produced by plants.

"Physiology" or the term "physiological characteristic" or a similar term will, when used herein, be understood to refer to the functional processes of an organism, including developmental processes such as growth, expansion and differentiation, sexual development, sexual reproduction, and in case of plants : seed set, seed development, grain filling, asexual reproduction, cell division, dormancy, germination, light adaptation, photosynthesis, leaf expansion, fibre production, secondary growth or wood production, amongst others; responses of a plant to externally-applied factors such as metals, chemicals, hormones, growth factors, environment and environmental stress factors (eg. anoxia, hypoxia, high temperature, low temperature, dehydration, light, daylength, flooding, salt, heavy metals, amongst others), including adaptive responses of plants to said externally-applied factors.

Means for introducing recombinant DNA into plant tissue or cells include, but are not limited to, transformation using $CaCl_2$ and variations thereof, in particular the method described by Hanahan (1983), direct DNA uptake into protoplasts (Krens *et al*, 1982;

Paszkowski *et al*, 1984), PEG-mediated uptake to protoplasts (Armstrong *et al*, 1990) microparticle bombardment, electroporation (Fromm *et al.*, 1985), microinjection of DNA (Crossway *et al.*, 1986), microparticle bombardment of tissue explants or cells (Christou *et al*, 1988; Klein *et al.*, 1992), vacuum-infiltration of tissue with nucleic acid, or in the case of plants, T-DNA-mediated transfer from *Agrobacterium* to the plant tissue as described essentially by An *et al.*(1985), Dodds *et al.* (1985), Herrera-Estrella *et al.* (1983a, 1983b, 1985).

For microparticle bombardment of cells, a microparticle is propelled into a cell to produce a transformed cell. Any suitable ballistic cell transformation methodology and apparatus can be used in performing the present invention. Exemplary apparatus and procedures are disclosed by Stomp *et al.* (U.S. Patent No. 5,122,466) and Sanford and Wolf (U.S. Patent No. 4,945,050). When using ballistic transformation procedures, the gene construct may incorporate a plasmid capable of replicating in the cell to be transformed.

Examples of microparticles suitable for use in such systems include 1 to 5 µm gold spheres. The DNA construct may be deposited on the microparticle by any suitable technique, such as by precipitation.

A whole plant may be regenerated from the transformed or transfected cell, in accordance with procedures well known in the art. Plant tissue capable of subsequent clonal propagation, whether by organogenesis or embryogenesis, may be transformed with a gene construct of the present invention and a whole plant regenerated therefrom. The particular tissue chosen will vary depending on the clonal propagation systems available for, and best suited to, the particular species being transformed. Exemplary tissue targets include leaf disks, pollen, embryos, cotyledons, hypocotyls, megagametophytes, callus tissue, existing meristematic tissue (e.g., apical meristem, axillary buds, and root meristems), and induced meristem tissue (e.g., cotyledon meristem and hypocotyl meristem).

The term "organogenesis", as used herein, means a process by which shoots and roots are developed sequentially from meristematic centres.

The term "embryogenesis", as used herein, means a process by which shoots and roots develop together in a concerted fashion (not sequentially), whether from somatic cells or gametes.

Preferably, the plant is produced according to the inventive method is transfected or transformed with a genetic sequence, or amenable to the introduction of a protein, by any art-recognized means, such as microprojectile bombardment, microinjection, *Agrobacterium*-mediated transformation (including *in planta* transformation), protoplast

fusion, or electroporation, amongst others. Most preferably said plant is produced by *Agrobacterium*-mediated transformation.

Agrobacterium-mediated transformation or agrolistic transformation of plants, yeast, moulds or filamentous fungi is based on the transfer of part of the transformation vector sequences, called the T-DNA, to the nucleus and on integration of said T-DNA in the genome of said eukaryote.

With "*Agrobacterium*" is meant a member of the *Agrobacteriaceae*, more preferably *Agrobacterium* or *Rhizobacterium* and most preferably *Agrobacterium tumefaciens*.

With "T-DNA", or transferred DNA, is meant that part of the transformation vector flanked by T-DNA borders which is, after activation of the *Agrobacterium vir* genes, nicked at the T-DNA borders and is transferred as a single stranded DNA to the nucleus of an eukaryotic cell.

When used herein, with "T-DNA borders", "T-DNA border region", or "border region" are meant either right T-DNA border (RB) or left T-DNA border (LB). Such a border comprises a core sequence flanked by a border inner region as part of the T-DNA flanking the border and/or a border outer region as part of the vector backbone flanking the border. The core sequences comprise 22 bp in case of octopine-type vectors and 25 bp in case of nopaline-type vectors. The core sequences in the right border region and left border region form imperfect repeats. Border core sequences are indispensable for recognition and processing by the *Agrobacterium* nicking complex consisting of at least VirD1 and VirD2. Core sequences flanking a T-DNA are sufficient to promote transfer of said T-DNA. However, efficiency of transformation using transformation vectors carrying said T-DNA solely flanked by said core sequences is low. Border inner and outer regions are known to modulate efficiency of T-DNA transfer (Wang et al. 1987). One element enhancing T-DNA transfer has been characterized and resides in the right border outer region and is called *overdrive* (Peralta et al. 1986, van Haaren et al. 1987).

With "T-DNA transformation vector" or "T-DNA vector" is meant any vector encompassing a T-DNA sequence flanked by a right and left T-DNA border consisting of at least the right and left border core sequences, respectively, and used for transformation of any eukaryotic cell.

With "T-DNA vector backbone sequence" or "T-DNA vector backbone sequences" is meant all DNA of a T-DNA containing vector that lies outside of the T-DNA borders and, more specifically, outside the nicking sites of the border core imperfect repeats.

The current invention includes optimized T-DNA vectors such that vector backbone integration in the genome of a eukaryotic cell is minimized or absent. With "optimized

T-DNA vector" is meant a T-DNA vector designed either to decrease or abolish transfer of vector backbone sequences to the genome of a eukaryotic cell. Such T-DNA vectors are known to the one familiar with the art and include those described by Hanson *et al.* (1999) and by Stuiver *et al.* (1999 - WO9901563).

With "binary transformation vector" is meant a T-DNA transformation vector comprising:

(a) a T-DNA region comprising at least one gene construct of interest and/or at least one selectable marker active in the eukaryotic cell to be transformed; and

(b) a vector backbone region comprising at least origins of replication active in *E. coli* and *Agrobacterium* and markers for selection in *E. coli* and *Agrobacterium*.

The T-DNA borders of a binary transformation vector can be derived from octopine-type or nopaline-type Ti plasmids or from both. The T-DNA of a binary vector is only transferred to a eukaryotic cell in conjunction with a helper plasmid.

With "helper plasmid" is meant a plasmid that is stably maintained in *Agrobacterium* and is at least carrying the set of *vir* genes necessary for enabling transfer of the T-DNA. Said set of *vir* genes can be derived from either octopine-type or nopaline-type Ti plasmids or from both.

With "super-binary transformation vector" is meant a binary transformation vector additionally carrying in the vector backbone region a *vir* region of the Ti plasmid pTiBo542 of the super-virulent *A. tumefaciens* strain A281 (EP0604662, EP0687730). Super-binary transformation vectors are used in conjunction with a helper plasmid.

With "co-integrate transformation vector" is meant a T-DNA vector at least comprising:

(a) a T-DNA region comprising at least one gene construct of interest and/or at least one selectable marker active in plants; and

(b) a vector backbone region comprising at least origins of replication active in *Escherichia coli* and *Agrobacterium,* and markers for selection in *E. coli* and *Agrobacterium,* and a set of *vir* genes necessary for enabling transfer of the T-DNA.

The T-DNA borders and said set of *vir* genes of a said T-DNA vector can be derived from either octopine-type or nopaline-type Ti plasmids or from both.

With "Ri-derived plant transformation vector" is meant a binary transformation vector in which the T-DNA borders are derived from a Ti plasmid and said binary transformation vector being used in conjunction with a 'helper' Ri-plasmid carrying the necessary set of *vir* genes.

As used herein, the term "selectable marker gene" or "selectable marker" or "marker for selection" includes any gene which confers a phenotype on a cell in which it is expressed to facilitate the identification and/or selection of cells which are transfected or transformed with a gene construct of the invention or a derivative thereof. Suitable selectable marker genes contemplated herein include the ampicillin resistance (Amp^r), tetracycline resistance gene (Tc^r), bacterial kanamycin resistance gene (Kan^r), phosphinothricin resistance gene, neomycin phosphotransferase gene (*nptII*), hygromycin resistance gene, β-glucuronidase (GUS) gene, chloramphenicol acetyltransferase (CAT) gene, green fluorescent protein (*gfp*) gene (Haseloff *et al*, 1997), and luciferase gene, amongst others.

With "agrolistics", "agrolistic transformation" or "agrolistic transfer" is meant here a transformation method combining features of *Agrobacterium*-mediated transformation and of biolistic DNA delivery. As such, a T-DNA containing target plasmid is co-delivered with DNA/RNA enabling in planta production of VirD1 and VirD2 with or without VirE2 (Hansen and Chilton 1996; *Hansen et al.* 1997; Hansen and Chilton 1997 - WO9712046).

Methods for transforming other organisms than plants such as funcgi, yeasts, insect cells, nematodes, animals and human cells are known in the art.

The term "cell cycle" means the cyclic biochemical and structural events associated with growth and with division of cells, and in particular with the regulation of the replication of DNA and mitosis. Cell cycle includes phases called: G0, Gap1 (G1), DNA synthesis (S), Gap2 (G2), and mitosis (M). Normally these four phases occur sequentially, however, the cell cycle also includes modified cycles wherein one or more phases are absent resulting in modified cell cycle such as endomitosis, acytokinesis, polyploidy, polyteny, and endoreduplication.

The term "cell cycle interacting protein", "cell cycle protein" or "cell cycle control protein" as denoted herein means a protein which exerts control on or regulates or is required for the cell cycle or part thereof of a cell, tissue, organ or whole organism and/or DNA replication. It may also be capable of binding to, regulating or being regulated by cyclin dependent kinases or their subunits. The term also includes peptides, polypeptides, fragments, variant, homologs, alleles or precursors (eg preproproteins or preproteins) thereof.

Cell cycle control proteins and their role in regulating the cell cycle of eukaryotic organisms are reviewed in detail by John (1981) and the contributing papers therein (Norbury and Nurse 1992;Nurse 1990;Ormrod and Francis 1993) and the contributing

papers therein (Doerner *et al.* 1996;Elledge 1996;Francis and Halford 1995;Francis *et al.* 1998;Hirt *et al.* 1991;Mironov *et al.* 1999) which are incorporated by reference.

The term "cell cycle control genes" refers to any gene or mutant thereof which exerts control on or are required for: chromosomal DNA synthesis and for mitosis (preprophase band, nuclear envelope, spindle formation, chromosome condensation, chromosome segregation, formation of new nuclei, formation of phragmoplast, duplication of microtubule-organizing center, etc) meiosis, cytokinesis, cell growth, endoreduplication, cell cycle control genes are also all genes exerting control on the above: homologues of CDKs, cyclins, E2Fs, Rb, CKI, Cks, and also any genes which interfere with the above, cyclin D, cdc25, Wee1, Nim1, MAP kinases, etc.

More specifically, cell cycle control genes are all genes involved in the control of entry and progression through S phase. They include, not exclusively, genes expressing "cell cycle control proteins" such as cyclin dependent kinases (CDK), cyclin dependent kinase inhibitors (CKI), D, E and A cyclins, E2F and DP transcription factors, pocket proteins, CDC7/DBF4 kinase, CDC6, MCM2-7, Orc proteins, cdc45, components of SCF ubiquitin ligase, PCNA, DNA-polymerase.

The term "cell cycle control protein" include cyclins A, B, C, D and E including CYCA1;1, CYCA2;1, CYCA3;1, CYCB1;1, CYCB1;2, CYC B2;2, CYCD1;1, CYCD2;1, CYCD3;1, and CYCD4;1 (Evans *et al.* 1983; Francis *et al.* 1998;Labbe *et al.* 1989;Murray and Kirschner 1989;Renaudin *et al.* 1996;Soni *et al.* 1995;Sorrell *et al.* 1999;Swenson *et al.* 1986) cyclin dependent kinase inhibitor (CKI) proteins such as ICK1 (Wang *et al.* 1997), FL39, FL66, FL67 (PCT/EP98/05895), Sic1, Far1, Rum1, p21, p27, p57, p16, p15, p18, p19 (Elledge 1996;Pines 1995), p14 and p14ARF; p13suc1 or CKS1At (De Veylder *et al.* 1997;Hayles and Nurse 1986) and nim-1 (Russell and Nurse 1987a;Russell and Nurse 1987b;Fantes 1989;Russell and Nurse 1986;Russell and Nurse 1987a;Russell and Nurse 1987b) homologues of Cdc2 such as Cdc2MsB (Hirt *et al.* 1993) CdcMs kinase (Bogre *et al.* 1997) cdc2 T14Y15 phosphatases such as Cdc25 protein phosphatase or p80cdc25 (Bell *et al.* 1993;Elledge 1996;Kumagai and Dunphy 1991;Russell and Nurse 1986) and Pyp3 (Elledge 1996) cdc2 protein kinase or p34cdc2 (Colasanti et al. 1991;Feiler and Jacobs 1990;Hirt *et al.* 1991;John et al. 1989;Lee and Nurse 1987;Nurse and Bissett 1981;Ormrod and Francis 1993) cdc2a protein kinase (Hemerly *et al.* 1993) cdc2 T14Y15 kinases such as wee1 or p107wee1 (Elledge 1996;Russell and Nurse 1986;Russell and Nurse 1987a;Russell and Nurse 1987b;Sun *et al.* 1999) mik1 (Lundgren *et al.* 1991) and myt1 (Elledge 1996); cdc2 T161 kinases such as Cak and Civ (Elledge 1996); cdc2 T161 phosphatases such as Kap1 (Elledge 1996); cdc28

protein kinase or p34cdc28 (Nasmyth 1993;Reed *et al.* 1985) p40MO15 (Fesquet *et al.* 1993;Poon *et al.* 1993) chk1 kinase (Zeng *et al.* 1998) cds1 kinase (Zeng *et al.* 1998) growth-associated H1 kinase (Labbe *et al.* 1989;Lake and Salzman 1972;Langan 1978;Zeng *et al.* 1998) MAP kinases described by (Binarova *et al.* 1998;Bögre et al. 1999;Calderini *et al.* 1998;Wilson *et al.* 1999).

Other cell cycle control proteins that are involved in cyclin D-mediated entry of cells into G1 from G0 include pRb (Xie *et al.* 1996;Huntley *et al.* 1998) E2F, RIP, MCM7 and potentially the pRb-like proteins p107 and p130.

Other cell cycle control proteins that are involved in the formation of a pre-replicative complex at one or more origins of replication, such as, but not limited to, ORC, CDC6, CDC14, RPA and MCM proteins or in the regulation of formation of this pre-replicative complex, such as, but not limited to, the CDC7, DBF4 and MBF proteins.

For the present purpose, the term "cell cycle control protein" shall further be taken to include any one or more of those proteins that are involved in the turnover of any other cell cycle control protein, or in regulating the half-life of said other cell cycle control protein. The term "protein turnover" is to include all biochemical modifications of a protein leading to the physical or functional removal of said protein. Although not limited to these, examples of such modifications are phosphorylation, ubiquitination and proteolysis. Particularly preferred proteins which are involved in the proteolysis of one or more of any other of the above-mentioned cell cycle control proteins include the yeast-derived and animal-derived proteins, Skp1, Skp2, Rub1, Cdc20, cullins, CDC23, CDC27, CDC16, and plant-derived homologues thereof (Cohen-Fix and Koshland 1997;Hochstrasser 1998;Krek 1998;Lisztwan *et al.* 1998) and Plesse et al in (Francis *et al.* 1998)).

For the present purpose, the term "cell cycle control genes" shall further be taken to include any one or more of those gene that are involved in the transcriptional regulation of cell cycle control gene expression such as transcription factors and upstream signal proteins. Additional cell cycle control genes are not excluded.

For the present purpose, the term "cell cycle control genes" shall further be taken to include any cell cycle control gene or mutant thereof, which is affected by environmental signals such as for instance stress, nutrients, pathogens, or by intrinsic signals such as the animal mitogens or the plant hormones (auxins, cytokinins, ethylene, gibberellic acid, abscisic acid and brassinosteroids).

The term "cell cycle progression" refers to the process of passing through the different cell cycle phases. The term "cell cycle progression rate" accordingly refers to the speed

at which said cell cycle phases are run through or the time spans required to complete said cell cycle phases.

The following figures and examples as described below are given for illustrative and non-limiting purposes only.

5

FIGURE LEGENDS

Figure 1. Schematical map of the pcosup-version3 T-DNA plant transformation vector. The T-DNA comprises the *npt*II selectable marker gene cassette and the GUS gene fragment extended by a stretch of 100 thymidine residues.

Figure 2. Schematical representation of the fragment of the pcosup-version T-DNA plant transformation vectors comprising the GUS gene fragment extended by a stretch of 50 thymidine residues (pcosup-version2) or 100 thymidine residues (pcosup-version3). For control purposes, pcosup-version1 is devoid of the thymidine stretch.

Figure 3. Schematic representation of the fragment of the pcosup-version4 plasmid (also designated p0481), comprising the GUS gene fragment extended by a stretch of 68 thymidine residues.

Figure 4. Schematic representation of results obtained from fluorimetric MUG assays. Numbers on the horizontal axis indicate independently transformed rice calli transformed with the plasmid indicated (pinterm1 or p0481). Values on the vertical axis indicate GUS activity (units/mg total protein). Panel A and B represent two independent experiments performed using different dilutions. Therefore, absolute values of GUS activity cannot be compared between these two experiments. MUG assays were performed according to standard procedures (Jefferson *et al.* 1987; Jefferson 1989) using total protein extracted from transgenic rice callus tissue.

EXAMPLES

Example 1. Construction of gene silencing vectors.

All recombinant DNA techniques were performed basically as outlined in Sambrook *et al.* (1989), Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory Press, NY) or in Ausubel *et al.* (1999), Current Protocols in Molecular Biology, CD-ROM (John Wiley & Sons Inc., NY).

Step 1: From the parent plasmid p0130 (CropDesign collection), the chimeric *npt*II gene cassette (pNOS promoter - *npt*II coding region - tOCS terminator) was isolated as a *Bam*HI fragment. The fragment was made blunt-end by filling in the overhangs with Klenow polymerase. A second parent plasmid p0216 (CropDesign collection) was digested with *Hin*dIII and *Eco*RI. The linear vector backbone was isolated and the overhangs filled in with Klenow polymerase. The resulting *npt*II gene cassette was ligated into the resulting vector backbone thus creating plasmid p0321 in which the *npt*II gene cassette was flanked at both sides by *Asc*I restriction sites.

Step 2: The rice GOS2 promoter (de Pater *et al.* 1992, Hensgens *et al.* 1993) was isolated as a *Xba*I-*Nco*I fragment from parent plasmid p0106 (CropDesign collection) and the overhangs filled in with Klenow polymerase. The resulting GOS2 promoter fragment was subsequently cloned into the unique *Pml*I-site of parent plasmid p0319 (CropDesign collection). Thus the plasmid p0368 was created in which the GOS2 promoter is flanked upstream by the T-DNA left border repeat (LB) and downstream by the T-DNA right border repeat (RB). In p0368 there is a unique *Asc*I-site in between the LB and the GOS2 promoter and a bidirect terminator sequence is located between the GOS2 promoter and the RB.

Step 3: The *npt*II expression cassette of p0321 (see step 1) was isolated as an *Asc*I fragment and inserted in the *Asc*I site of p0368 (see step 2), thus creating the plasmid pinterm1.

Step 4: By means of PCR, a truncated β-glucuronidase (GUS) gene which contains a catalase intron was obtained and purified. As a template, parent plasmid p0106 (CropDesign collection) was used. The sense primer consisted of the following nucleotide sequence (primer tm1: 5'-ATGGTAGATCTGAGGGTAAAT-3'; SEQ ID NO 1).

Three different antisense primers were used:

- primer tm2 (5'-TTCATAGAGATAACCTTCACC-3'; SEQ ID NO2) such that, in combination with tm1 only the truncated GUS gene fragment is amplified;
- primer tm3 (5'-AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA-AAAAAATTCATAGAGATAACCTTCACC-3'; SEQ ID NO3) containing a stretch of 50 adenosine nucleotides such that, in combination with tm1 the truncated GUS gene fragment is amplified and extended with a stretch of 50 thymidine nucleotides;
- primer tm4 (5'-AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA-AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA ATTCATAGAGATAACCTTCACC-3'; SEQ ID NO4) containing a stretch of 100 adenosine nucleotides such that, in combination with tm1 the truncated GUS gene fragment is amplified and extended with a stretch of 100 thymidine nucleotides.

Step 5: The PCR fragments obtained in Step 4 were subsequently inserted in the *Swa*I-site of pinterm1 (see step 3) such that the truncated GUS gene fragment is downstream of the GOS2 promoter and upstream of the bidirect terminator derived from the Ti-plasmid.

The three resulting plasmids are denominated as:

- pcosup-version1 containing the GOS2 promoter, the truncated GUS gene fragment and the bidirect terminator;
- pcosup-version2, containing the GOS2 promoter, the truncated GUS gene fragment extended by 50 thymidine nucleotides and the bidirect terminator;
- pcosup-version3, containing the GOS2 promoter, the truncated GUS gene fragment extended by 100 thymidine nucleotides and the bidirect terminator.

As an illustration, pcosup-version3 is schematically depicted in Figure 1. The spatial organisation of the truncated GUS gene fragment, the poly-T-stretch and the bidirect terminator is enlarged in Figure 2.

Example 2. *Agrobacterium*-mediated rice transformation

Constructs outlined in Example 1 are transformed to *Agrobacterium tumefaciens* strain LBA4404 or C58 by means of electroporation and transformed bacterial cells selected on a solid agar medium containing the appropriate antibiotics.

For demonstration of HDTS using the gene silencing vectors of the current invention, rice plants that express a transgene comprising the full-length GUS gene are used. The

plant transformation vectors pcosup-version2 and pcosup-version3, both containing a poly-T-stretch according to the invention, are used to demonstrate HDTS whereas plant-transformation vector pcosup-version1 is used as a control.

Mature dry seeds of the rice japonica cultivars Nipponbare or Taipei 309 are dehusked, sterilised and germinated on a medium containing 2,4-D (2,4-dichlorophenoxyacetic acid). After incubation in the dark for four weeks, embryogenic, scutellum-derived calli are excised and propagated on the same medium. Selected embryogenic callus is then co-cultivated with *Agrobacterium*. Co-cultivated callus is grown on 2,4-D-containing medium for 4 to 5 weeks in the dark in the presence of a suitable concentration of the appropriate selective agent. During this period, rapidly growing resistant callus islands develop. After transfer of this material to a medium with a reduced concentration of 2,4-D and incubation in the light, the embryogenic potential is released and shoots develop in the next four to five weeks. Shoots are excised from the callus and incubated for one week on an auxin-containing medium from which they can be transferred to the soil. Hardened shoots are grown under high humidity and short days in a phytotron. Seeds can be harvested three to five months after transplanting. The method yields single locus transformants at a rate of over 50 % (Aldemita and Hodges1996, Chan et al. 1993, Hiei et al. 1994).

Example 3. Analysis of HDTS

Untransformed rice plants and rice plants transformed with pcosup-version1 serve as controls for measuring GUS activity in crude protein extracts prepared from rice leaves. In both cases GUS activity is exerted by the full-length GUS gene cassette already resident in the rice plants. The truncated GUS gene fragment of the pcosup-version vectors is not contributing to GUS activity. In rice plants transformed with pcosup-version1 or pcosup-version2, the endogenous GUS-activity is lowered due to gene silencing effects brought about by said vectors.

GUS activity can easily be measured by colorometric/fluorometric assays using GUS substrates such as 4-methylumbelliferyl β-D-glucuronide (MUG) or 5-bromo-4-chloro 3-indolyl β-D-glucuronide (X-GLUC); reference is made to Jefferson *et al.* 1987 and Jefferson 1989.

Example 4. Transformation of GUS expressing rice calli with a gene silencing vector containing a truncated GUS gene extended with a polyT tail of 68 nucleotides results in high frequency of GUS gene silencing.

A pcosup-version4 plasmid was constructed similar to pcosup-version3 as described in Example 1. The plasmids pcosup-version3 and pcosup-version4 are similar except that that the polyT tail in pcosup-version4 is 68 thymidine nucleotides long. As an illustration, the part of the pcosup-version4 plasmid containing the truncated GUS gene followed by the 68 nucleotide T-tail is schematically depicted in Figure 3. The pcosup-version4 plasmid is also designated p0481 as denoted herein. p0481 was used for transformation of transgenic rice callus that expressed the GUS gene as described in Example 2. As a control, the transgenic rice callus was also transformed with the empty vector construct pinterm1 (see Example 1, step 3).

GUS activity of rice callus transformed with pinterm1 and p0481 was determined by fluorimetric assays using the substrate 4-methylumbelliferyl β-D-glucuronide (MUG) according to established procedures (Jefferson *et al.* 1987; Jefferson 1989).

The results of these MUG assays are shown in Figure 4A and 4B. Figure 4A and 4B represent data from two independent MUG assays performeded on calli derived from independent transformation events. GUS activity is expressed in units per microgram total protein extract. The absolute values cannot be compared between the two data sets (A and B). As shown in Figure 4, GUS activity was high for all 8 calli transformed with pinterm1. This was expected since the empty vector construct pinterm1 should not induce silencing of the resident GUS gene. By contrast, calli transformed with p0481 frequently showed reduced or highly reduced GUS gene silencing: reduced GUS activity was observed for lines 3, 8 and 9 of the first dataset (Figure 4A) and for lines 3, 16, 17 in the second dataset (Figure 4B); highly reduced GUS activity was observed in lines 1, 2, 4, 7, and 10 of the first dataset (Figure 4A) and in lines 1, 2, 4, 5, and 7 through 15 of the second dataset (Figure 4B). Therefore, it is disclosed in this invention that the GUS construct with the polyT insert induced highly efficient silencing of the resident GUS gene.

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CLAIMS

1. A nucleic acid construct (transgene) for downregulating the expression of a target nucleic acid in a cell, organism or tissue, comprising the following operably linked elements :
   a) a promoter capable of driving expression of said nucleic acid in said cell, organism or tissue,
   b) a transcribed region of which at least part of the generated transcript is homologous to either the sense or antisense transcript of the targeted nucleic acid,
   c) a DNA sequence within the transcribed region of said nucleic acid which consists of a stretch of T bases in the transcribed strand, and,
   d) a DNA sequence immediately following the poly-T stretch which causes transcript termination.
2. A nucleic acid construct of claim 1 wherein said DNA sequence of c) comprises a stretch of T bases of 500 nucleotides or less.
3. A vector comprising a nucleic acid construct (transgene) of claim 1.
4. A vector of claim 3 suitable for high-throughput cloning of target nucleic acid sequences, comprising a cloning site for inserting any desired target nucleic acid or a part thereof.
5. A host cell comprising a vector according to claim 3 or 4.
6. The host cell of claim 5, wherein said host cell is a plant, insect, nematode, fungal, plant, human or animal cell.
7. A method for downregulating the expression of a target nucleic acid in an organism, tissue or cell comprising the introduction of a vector of claim 3 or 4 into said organism, tissue or cell, resulting in the expression of said nucleic acid construct of claim 1 or 2.
8. The method of claim 7 wherein said nucleotide sequence is stably introduced into the genome of said organism or cell.
9. The method according to claim 7 or 8 wherein said cell is a plant cell.
10. The method of claim 9 further comprising regenerating a plant from said plant cell.
11. A method for the production of transgenic plants, plant cells or plant tissues comprising the introduction of a vector of any of claim 3 or 4 in an expressible format in said plant, plant cell or plant tissue.

12. A transgenic plant cell comprising a nucleic acid construct of claim 1 or 2 or obtainable by a method of claim 10 or 11.

13. The transgenic plant cell of claim 12 wherein said nucleic acid construct of claim 1 or 2 is stably integrated into the genome of said plant cell.

14. A transgenic plant or plant tissue comprising plant cells of claim 12 or 13 or obtainable by a method of claim 11.

15. A harvestable part or extract or derivative of a plant of claim 14.

16. The harvestable part of claim 15 which is selected from the group consisting of seeds, fruits, leaves, stem cultures, rhizomes and bulbs.

17. The progeny derived from any of the plants or plant parts of any of claims 14 to 16.

18. A method for modifying cell fate comprising the use of a method of any of claims 7 to 11.

19. A method for modifying the development of a whole organism, tissue or organ comprising the use of a method of any of claims 7 to 11.

20. A method for modifying the morphology of a whole organism, tissue or organ comprising the use of a method of any of claims 7 to 11.

21. A method for modifying the biochemistry of a whole organism, tissue or organ comprising the use of a method of any of claims 7 to 11.

22. A method for modifying the physiology of a whole organism, tissue or organ comprising the use of a method of any of claims 7 to 11.

23. A method for modifying the cell cycle progression rate of a whole organism, tissue or organ comprising the use of a method of any of claims 7 to 11.

24. A method for obtaining enhanced growth of a whole organism, tissue or organ comprising the use of a method of any of claims 7 to 11.

25. A method for obtaining increased yield of a plant or harvestable part thereof comprising the use of a method of any of claims 7 to 11.

26. A method for obtaining delayed senescence of a whole plant, a plant tissue or organ comprising the use of a method of any of claims 7 to 11.

27. A method for modifying the content of desaturated lipids of a plant by downregulating a fatty acid desaturase comprising the use of a method of any of claims 7 to 11.

28. A method for improving fruit handling characterstics by downregulating fruit ripening genes comprising the use of a method of any of claims 7 to 11.

29. A method for elimination of toxic secondary metabolites in a plant by downregulating a biosynthetic gene comprising the use of a method of any of claims 7 to 11.

30. A method for promoting and extending cell division activity in cells or organisms in adverse conditions and/or consequences of stress, comprising the use of a method of any of claims 7 to 11.

31. A method for conferring enhanced resistance to pathogens comprising the use of a method of any of claims 7 to 11.

32. A method for conferring enhanced resistance to pathogens causing neoplastic growth comprising the use of a method of any of claims 7 to 11.

33. A method for increasing seed yield and/or seed size comprising the use of a method of any of claims 7 to 11.

34. A method for identifying the function of a target nucleic acid in an organism, cell, tissue or organ, comprising elucidating the phenotype of the organism, cell, organ or tissue which transcribe a nucleic acid construct of claim 1 or 2.

35. A method of claim 34 wherein said target nucleic acid is a gene, a cDNA or a part of said gene or cDNA.

36. A method of claim 34 or 35 further characterized as being a high-throughput cloning method for identifying the function of target nucleic acids, comprising the use of a vector of claim 4.

37. The method of any of claims 34 to 36 wherein said organism is a plant, insect, nematode, fungus, or a non-human animal.

38. Use of a nucleic construct of claim 1 or 2 or a vector of claim 3 or 4 for modifying cell fate.

39. Use of a nucleic construct of claim 1 or 2 or a vector of claim 3 or 4 for modifying development of an organism.

40. Use of a nucleic acid construct of claim 1 or 2 or a vector of claim 3 or 4, for modifying morphology of an organism.

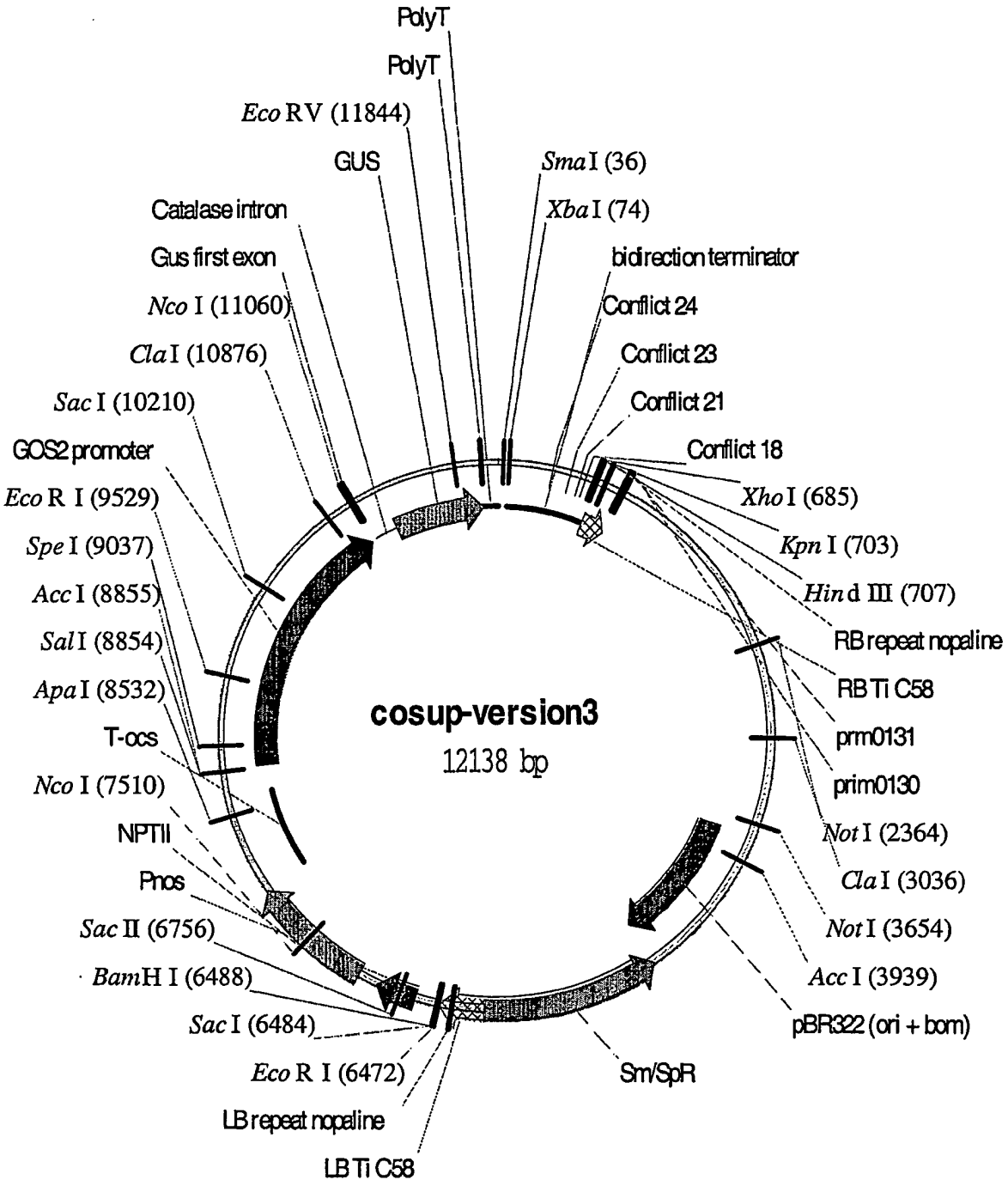
41. Use of a nucleic acid construct of claim 1 or 2 or a vector of claim 3 or 4 for modifying plant biochemistry.

42. Use of a nucleic acid construct of any claim 1 or 2 or a vector of claim 3 or 4 for modifying plant physiology.

43. A nucleic acid construct of claim 1 or 2 or a vector of claim 3 for use as a medicament.

44. Use of nucleic acid construct of claim 1 or 2 or a vector of claim 3 for the preparation of a medicament for treating infectious diseases, tumors, auto-immune diseases, diseases due to organ gland or tissue malfunctioning, diseases due to biochemical malfunctioning, transplant rejection reactions or septic shock in animals or humans.

45. A composition comprising a nucleic acid construct of claim 1 or 2 or a vector of claim 3 or a host cell of claim 5 or 6.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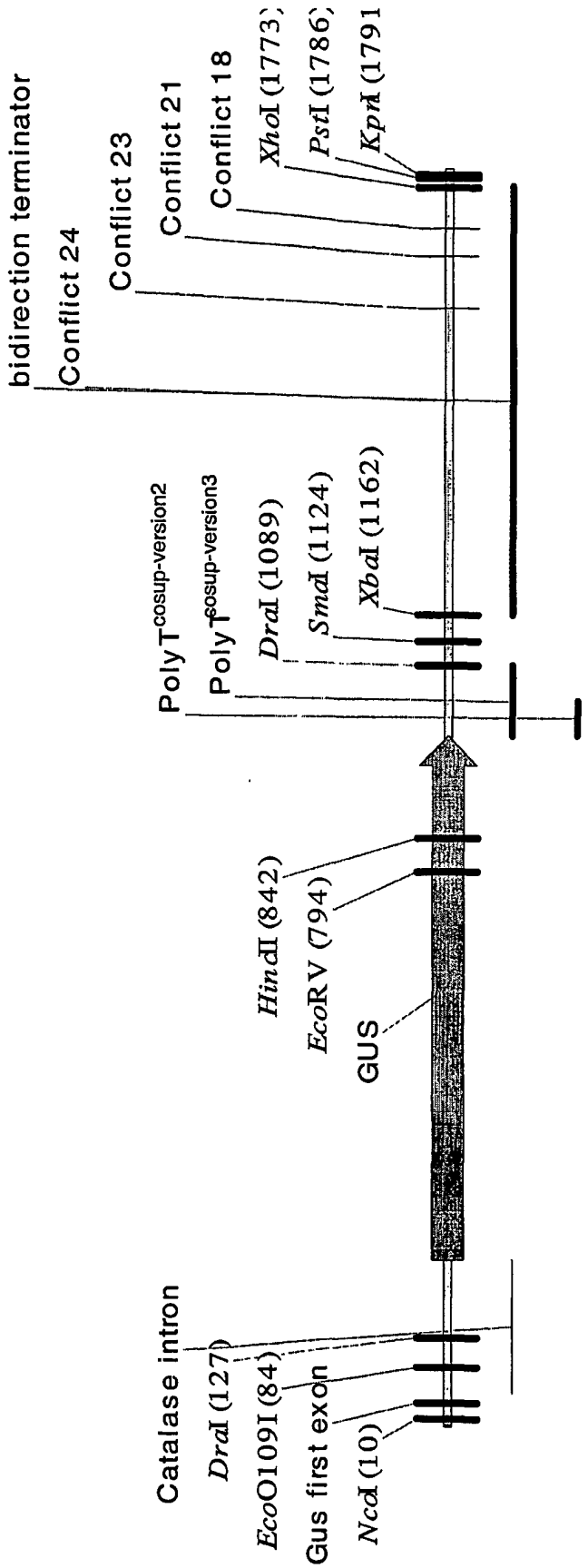

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FIGURE 2

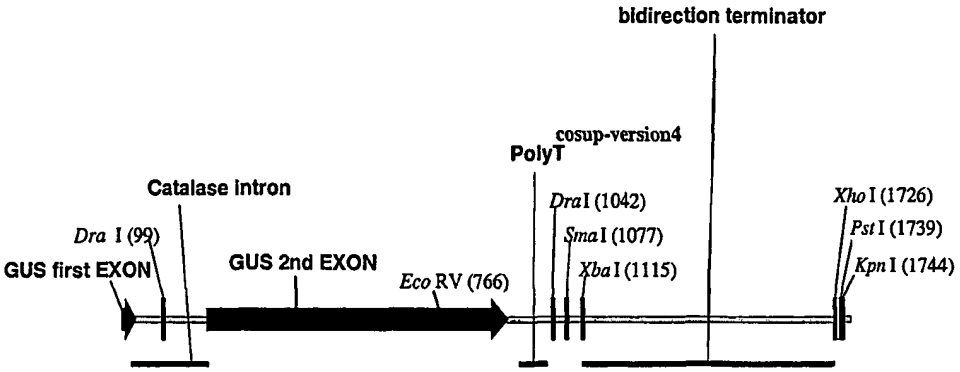

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Figure 3

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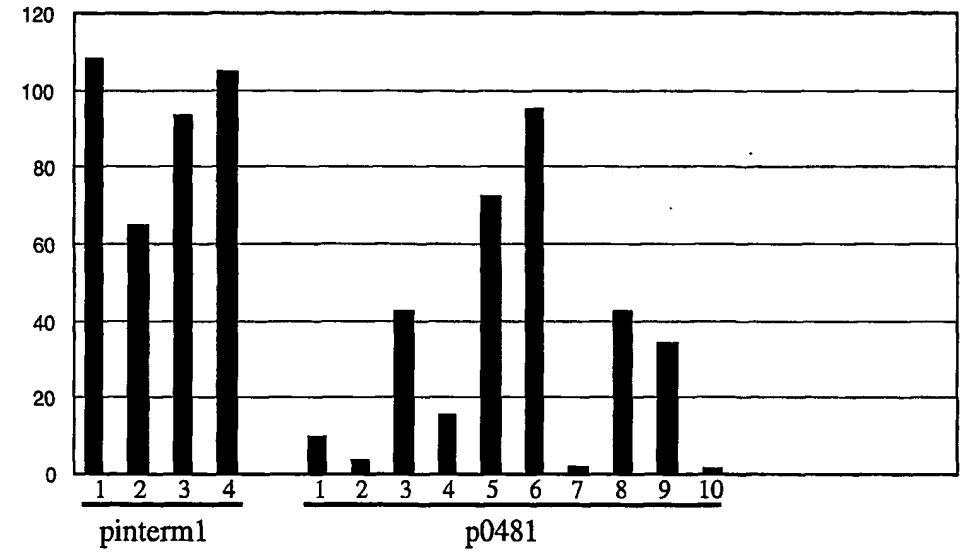
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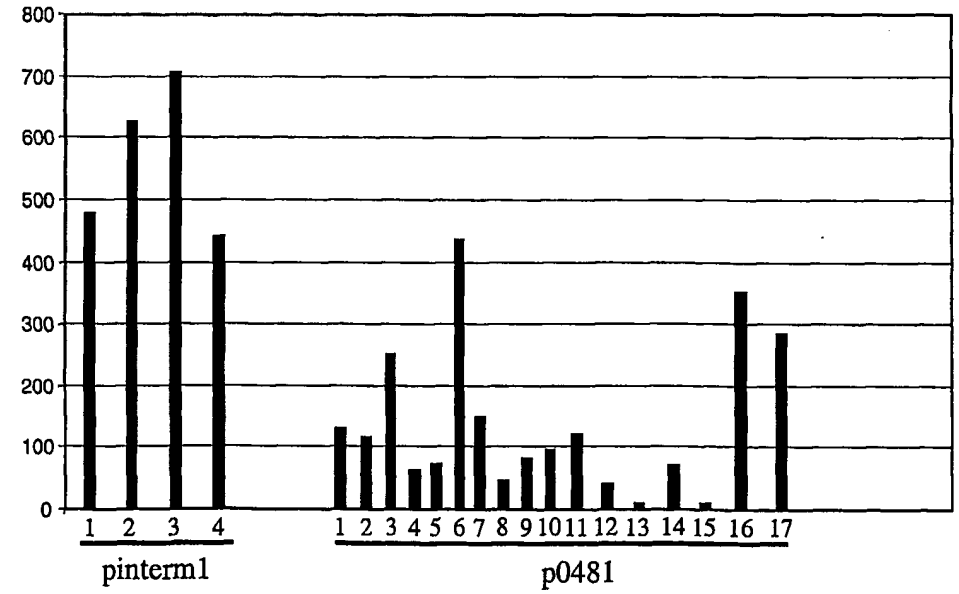
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Figure 4

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